Activity of *Pseudomonas aeruginosa* in Biofilms: Steady State

R. Bakke, M. G. Trulear,* J. A. Robinson, and W. G. Characklis
Institute for Biological and Chemical Process Analysis, Montana State University, Bozeman, Montana 59717

Accepted for Publication March 22, 1984

Aerobic glucose metabolism by *Pseudomonas aeruginosa* in steady-state biofilms at various substrate loading rates and reactor dilution rates was investigated. Variables monitored were substrate (glucose), biofilm cellular density, biofilm extracellular polymeric substance (EPS) density, and suspended cellular and EPS concentrations. A mathematical model developed to describe the system was compared to experimental data. Intrinsic yield and rate coefficients included in the model were obtained from suspended continuous culture studies of glucose metabolism by *P. aeruginosa*. Experimental data compared well with the mathematical model, suggesting that *P. aeruginosa* does not behave differently in steady-state biofilm cultures, where diffusional resistance is negligible, than in suspended cultures. This implies that kinetic and stoichiometric coefficients for *P. aeruginosa* derived in suspended continuous culture can be used to describe steady-state biofilm processes.

INTRODUCTION

Biofilms are biologically active matrices of cells and noncellular material accumulated on solid surfaces. Predicting the rate and extent of biofilm processes would be useful in ecosystem analysis, design and operation of heat exchangers and pipelines subject to fouling, wastewater treatment plant design and operation, and determining the feasibility of biofilm reactors for biotechnological applications. However, only limited information is available on which to base phenomenological biofilm models.

This article reports results obtained with axenic steady-state biofilms composed of *Pseudomonas aeruginosa* and extracellular polymeric substances (EPS). The substrate, glucose, which was the rate-limiting nutrient and sole carbon and energy source, was fed continuously to the biofilm via the turbulent aqueous phase of the biofilm reactor. A previous article from our laboratory reported results of chemostat experiments with this organism, including stoichiometric and rate coefficients describing cellular reproduction and extracellular product formation in suspended culture.[1]

The major objective of this work was to determine the usefulness of stoichiometric and rate data obtained in suspended culture for describing steady-state biofilm processes. Navarro and Durand[2] summarized several studies in which it has been demonstrated that both stoichiometry and kinetics of various bacteria change following immobilization. Our results, however, demonstrate that the same kinetic and stoichiometric coefficients can be used to describe processes carried out by *P. aeruginosa* in suspended cultures as in biofilms.

Results from the non-steady-state biofilm accumulation phase preceding the steady state phase will be published in a separate report.

MATHEMATICAL DESCRIPTION OF REACTOR SYSTEM

The annular reactor (AR) was operated as a continuous flow stirred tank reactor (CFSTR) in which bulk liquid concentration gradients ideally do not exist (Fig. 1). Accumulation of compounds in the bulk liquid can be described by material balances of the general form:

$$\begin{matrix}\text{net}\\ \text{rate of}\\ \text{accumulation}\end{matrix} = \begin{matrix}\text{net}\\ \text{rate of}\\ \text{transport}\end{matrix} + \begin{matrix}\text{net}\\ \text{rate of}\\ \text{transformation}\end{matrix} \quad (1)$$

Accumulation of attached biofilm components can be described by constitutive equations of the same general form as eq. (1), but with no transport term. Material balances and constitutive equations are organized in matrix notation as described by Roels.[3]

The components analyzed in this study were bulk liquid substrate concentration, s, biofilm cellular areal density, x_b, biofilm EPS areal density, p_b, suspended cellular concentration, x, and suspended EPS concentration, p. The component vector, $\mathbf{a}$, is defined as:

$$\mathbf{a} = \begin{vmatrix} s \\ x_b \\ p_b \\ x \\ p \end{vmatrix} \quad (2)$$

The left-hand side of eq. (1) can then be described as $d\mathbf{a}/dt$.

*Present address: Nalco Chemical Company, Naperville, IL.

Biotechnology and Bioengineering, Vol. XXVI, Pp. 1418-1424 (1984)

CCC 0006-3592/84/121418-07$04.00

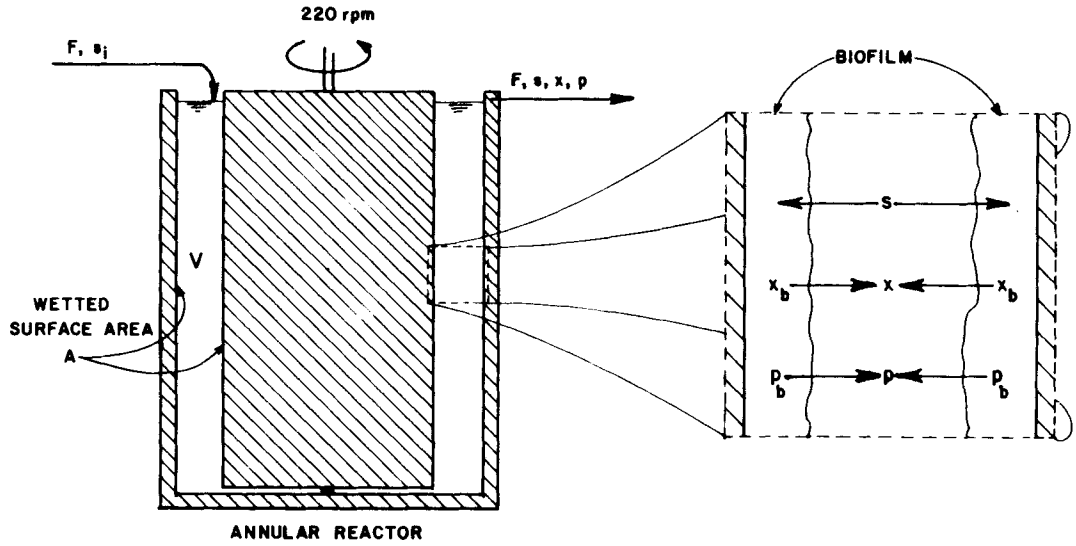

Figure 1. The annular reactor (AR) operated as a continuous flow stirred tank reactor (CFSTR). The insert describes a local environment in the reactor. The mathematical model was based on this conceptual model.

There is no transport term for the biofilm components and no input suspended biomass after the inoculation period. The transport vector, $\mathbf{a}'$, then becomes:

$$\mathbf{a}' = \begin{vmatrix} D(s_i - s) \\ 0 \\ 0 \\ -Dx \\ -Dp \end{vmatrix} \tag{3}$$

Stoichiometric relations [eqs. (4)-(7)] describing the processes presumed to be of significance to the analysis are listed in Table I. The yield coefficients represent stoichiometric ratios between the product (x or p) formed and the amount of reactant (s) consumed for that particular process and are, therefore, fundamentally characteristic of aerobic *P. aerunginosa* glucose metabolism. Observed yields, which represent the ratio between the product and the total amount of reactant consumed, do not have the fundamental significance of intrinsic yields and should not be confused with these. The reactors were operated at relatively high dilution rates ($D >> \mu_{max}$) so cellular reproduction and product formation by suspended organisms were negligible.

The extent of these transformation processes is determined by their reaction rates and the time allowed for the processes in the reactor, as described by eq. (1). Reaction rate expressions are listed in Table II [eqs. (8)-(11)]. The growth rate was presumed to be a saturation function of reactor substrate concentration as first suggested by Monod.[4] The expression is useful as long as minimal diffusional resistance exists as is the case in this study.[5] The EPS formation rate was found[5] to obey the Luedeking-Piret equation[6] for product formation. Biomass detachment from the biofilm was presumed independent of substrate load, growth rate, and dilution rate. Data suggest that specific biofilm mass detachment rate is a function of biofilm mass,[7] but the kinetic order of these functions have not been determined and was, therefore, expressed as a power-law function in this model.

Combining stoichiometry and kinetics in a transformation vector, $\mathbf{a}''$, yields:

$$\mathbf{a}'' = \begin{vmatrix} -\mu x_b / Y_{x/s} - q_p x_b / Y_{p/s} \\ \mu x_b - q_{dx} x_b \\ q_p x_b - q_{dp} p_b \\ q_{dx} x_b A / V \\ q_{dp} p_b A / V \end{vmatrix} \tag{12}$$

Equation (1) can now be expressed as

$$d\mathbf{a}/dt = \mathbf{a}' + \mathbf{a}'' \tag{13}$$

Complete material balances and constitutive equations

Table I. Stoichiometric equations for transformation processes in biofilm reactor.

Equation	Description	Process
(4)	growth	$s \rightarrow x_b (Y_{x/s})^{-1}$
(5)	product (EPS) formation	$s \rightarrow p_b (Y_{p/s})^{-1}$
(6)	cellular detachment	$x_b \rightarrow x$
(7)	EPS detachment	$p_b \rightarrow p$

Table II. Reaction rate expressions for transformation processes.

Equation	Description	Process
(8)	specific growth rate	$\mu = \mu_{max} s/(K_s + s)$
(9)	specific EPS formation rate	$q_p = k\mu + k'$
(10)	specific cellular detachment rate	$q_{dx} = k_{dx} x_b''$
(11)	specific EPS detachment rate	$q_{dp} = k_{dp} p_b''$

Table III. Material balances and constitutive equations for a biofilm continuous flow stirred tank reactor (annular reactor) in which substrate transformation by suspended cells is negligible.

Equation	Compound: Parameter	Net rate of accumulation	Net rate of transport out of reactor	Net rate of transformation	Units
(14)	bulk liquid substrate concentration, s	$ds/dt =$	$D(s_i - s)$	$-\left(\frac{\mu}{Y_{x/s}} + \frac{q_p}{Y_{p/s}}\right)x_b \frac{A}{V}$	$M/L^3/t$
(15)	biofilm cellular areal density, x_b	$dx_b/dt =$		$\mu x_b - q_{dx}x_b$	$M/L^2/t$
(16)	biofilm EPS areal density, p_b	$dp_b/dt =$		$q_p x_p - q_{dp}p_b$	$M/L^2/t$
(17)	suspended cellular mass, x	$dx/dt =$	$-Dx$	$+ q_{dx}x_b \frac{A}{V}$	$M/L^3/t$
(18)	suspended EPS mass, p	$dp/dt =$	$-Dp$	$+ q_{dp}p_b \frac{A}{V}$	$M/L^3/t$

derived from eq. (13) are listed in Table III [eqs. (14)-(18)].

Steady State

Biofilm accumulation reaches a plateau when $n > 0$ [eqs. (10) and (11)], i.e., $d\mathbf{a}/dt = 0$ or *steady state*. Equation (13) then simplifies to:

$$0 = \mathbf{a}' + \mathbf{a}'' \tag{19}$$

Transformation rates presented in Table II can then be determined from eq. (19) since all quantities in the transport vector are measurable [Table IV; eqs. (20)-(23)].

Substrate Removal

At steady state, $ds/dt = 0$ and the substrate material balance can be rearranged:

$$D(s_i - s)x_b^{-1}V/A = \mu(1/Y_{x/s} + k/Y_{p/s}) + k'/Y_{p/s} \tag{24}$$

Specific substrate removal rate, q_s, is defined as the left hand side of eq. (24):

$$q_s = D(s_i - s)x_b^{-1}V/A \tag{25}$$

Since yield coefficients represent reaction (as opposed to observed) stoichiometries for *P. aeruginosa*, they should be constant. Equation (25), therefore, predicts a linear relation between q_s and μ:

$$q_s = \mu(1/Y_{x/s} + k/Y_{p/s}) + k^1/Y_{p/s} \tag{26}$$

Table IV. Steady-state specific transformation rates. All quantities on the right-hand side are measured quantities.

Equation	Description	Process
(20)	cellular detachment	$q_{dx} = D(x/x_b)\,V/A$
(21)	growth	$\mu = q_{dx}$
(22)	EPS detachment	$q_{dp} = D(p/p_b)\,V/A$
(23)	EPS formation	$q_p = D(p/x_b)\,V/A$

The overall substrate flux across the liquid biofilm interface, η_s, is defined as:

$$\eta_s = D(s_i - s)V/A \tag{27}$$

Biofilm Areal Densities

Combining Equations 25 and 27 yields:

$$x_b = \eta_s/q_s \tag{28}$$

For constant q_s, eq. (28) predicts that biofilm cellular areal density should be proportional to η_s.

At steady state, eq. (16) becomes:

$$p_b = q_p x_b/q_{dp} \tag{29}$$

Liquid Phase Concentrations

At steady state, eq. (17) becomes:

$$x = x_b(q_{dx}/D)A/V \tag{30}$$

and eq. (18):

$$p = p_b(q_{dp}/D)A/V \tag{31}$$

Experimental results will be compared to the equations derived in this section to test the validity of the mathematical model and, therefore, its underlying assumptions. Stoichiometric and kinetic coefficients for aerobic glucose metabolism by *P. aeruginosa* in chemostats were used to determine the usefulness of data obtained in dispersed culture to describe biofilm processes.

METHODS

Experimental System

All experiments were conducted in an annular reactor (AR) system consisting of two to three AR's, sterile dilution water, and sterile substrate feed apparatus including temperature control (Fig. 2). The AR provided a wetted surface area to volume ratio, $A/V = 275\ m^{-1}$, and a turbulent bulk liquid phase. More details regarding the AR can be found elsewhere.[5,7,8]

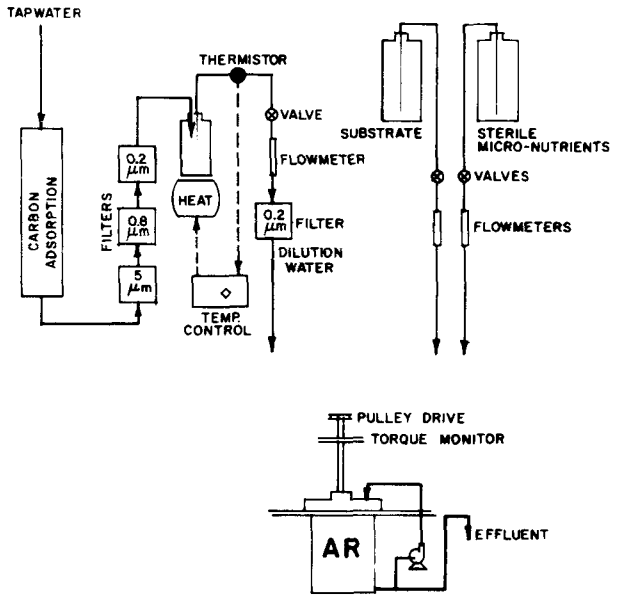

Figure 2. Annular reactor system.

Experimental Procedures

The AR's were cleaned, assembled, and then sterilized with hypochlorite solution (approximately 5 kg/m^3) prior to all experiments. Dilution water was sterilized by filtration (see Fig. 2) and the flow rate was adjusted. A minimum of 50 residence times was allowed for residual chlorine removal before inoculation. Experiments were started by pumping *P. aeruginosa* (AR feed = 5.6×10^8 cells/h) from a steady-state chemostat (dilution rate, $D = 0.075$ h^{-1}) into the AR for a period of 12 h (experiments 1 and D3) or 18 h (experiments 2-7), thus providing a defined condition for initial attachment and colonization. Autoclaved substrate and nutrient solutions were fed into the AR throughout each experiment, including the 12 or 18 h inoculation period.

A loopful of reactor solution was streaked on a glucose-micronutrient (GMN) agar plate and on a trypticase soy-yeast extract (TSY) agar plate during each experimental sampling period (at least once a day) to check for contamination using colonial morphology as an indicator. The API 20E system was also used to check for the presence of contaminants in the reactors. Experiments were terminated and reinitiated upon the detection of contaminants.

Analytical Methods

The hierarchy of analytical procedures performed on biofilm or reactor liquid samples is shown in Figure 3.

Glucose

Glucose concentration was determined with a modified version of the Sigma 510 Glucose Analysis procedure[5] (Sigma Chemical Co., St. Louis, MO).

Organic Carbon

Total organic carbon (TOC) was measured using the ampule analysis module of an Oceanography International (IO) carbon analyzer. Soluble organic carbon (SOC) was also measured using the IO carbon analyzer after filtering the sample through a Nuclepore filter (0.45 μm). The difference between TOC and SOC is particulate organic carbon (POC).

Cellular Carbon

Total cell number concentration was determined by enumerating cells stained with acridine orange using epifluorescence microscopy according to the methods of Hobbie and co-workers.[9] Cellular carbon was determined

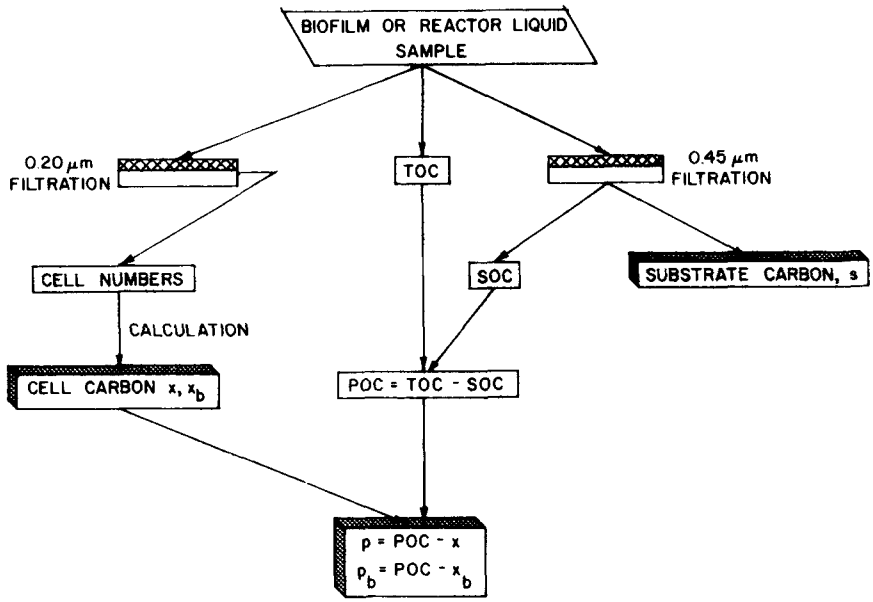

Figure 3. Hierarchy of analytical procedures.

as the product of the estimated average cell volume in each cell enumeration sample and the cell number,[1,5] multiplied by the following factors: 1.07 g cell/cm^3 [10,11] and 0.11 g C/g cell.[11,12]

Extracellular Polymeric Substances (EPS) Carbon

The EPS carbon was calculated as the difference between POC and cellular carbon.

RESULTS AND DISCUSSION

A summary of experimental conditions and steady-state results is presented in Tables V and VI. All mass measurements, including substrate, cells, and EPS, are reported in terms of carbon equivalents. Data are reported as the average of a minimum of two measurements for each sample. Reported error limits represent standard deviations from two to three reactors.

Coefficients characteristic of glucose metabolism by *P. aeruginosa* in a chemostat previously obtained in our laboratory[1] are shown in Table VII.

Specific Substrate Removal Rate, *r*

Equation (24) predicts a linear relationship between q_s and the specific cellular growth rate, μ. Biofilm data are compared to eq. (24) with coefficients determined in suspended cultures (Table VII) in Figure 4. Chemostat data from which the coefficients were determined are also included in Figure 4. A t-test was performed on the data to statistically determine the correlation between the biofilm data and the chemostat data. At the five percent level of statistical significance, there is no difference between the chemostat data and the combination of the biofilm and the chemostat data. This supports the contention that coefficients obtained for chemostat cultures may be used to predict biofilm kinetics. From these results, it is also clear that the estimated yield and rate coefficients are intrinsic quantities as opposed to observed quantities.

To emphasize the significance of biomass separation into two components, cell and EPS mass, a traditional analysis based on suspended solids data ($x + p$ and $x_b + p_b$) was also performed. The fit of substrate removal rate per total biofilm mass [$= q_s x_b/(x_b + p_b)$] versus the specific biomass production rate [$= \mu(x_b/x)\,(x + p)/(x_b + p_b)$] to eq. (24) is presented in Figure 5. The data are not consistent with eq. (24). The data presented in Figure 5 could have led to the erroneous conclusion that the metabolic activity in the biofilms was significantly different than in suspension.

Our mathematical model predicts that the y-intercept of the q_s vs. μ curve should be greater than zero and equal to $k'/Y_{p/s}$ (eq. (26)]. The y-intercept was significantly different from zero at the five percent level of statistical significance, implying that non-growth-associated EPS formation is significant. Maintenance requirements exhibited by *P. aeruginosa* in biofilms can, therefore, probably be attributed mainly to EPS formation, as was demonstrated to be the case in chemostats.[1]

Table VI. Summary of process rates at steady state.

Experiment No.	μ, q_{dx} (h^{-1})	q_s (g substrate/ (g cell h)	η_s (mg/m^{2}h)
1	0.163	0.61	33
2 and 7	0.198	0.71 ± 0.27	73
4 and 5	0.141	0.66 ± 0.21	338
6	0.137	0.53 ± 0.01	179
D3	0.119	0.53 ± 0.18	40

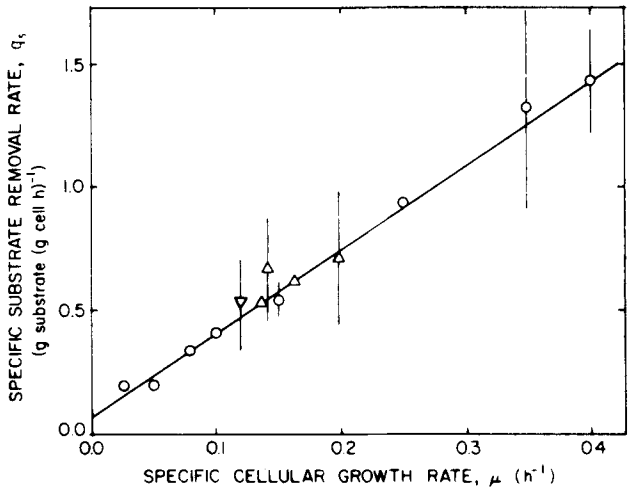

Figure 4. Steady-state biofilm q_s versus μ data: (△) $D = 6\ h^{-1}$; (▽) $D = 3\ h^{-1}$. The (○) line [eq. (24)] is based on chemostat data (ref. 1). The slope is 3.4 ± 0.1 (g substrate/g cell) and the intercept is 0.063 ± 0.022 (g substrate/g cell h). Error bars represent standard error of data from two or three reactors.

Table V. Experimental conditions and summary of experimental results at steady state.

Experiment No.	D (h^{-1})	No. of replications	s_i (g/m^3)	s (g/m^3)	x_b (mg/m^2)	p_b (mg/m^2)	x (g/m^3)
1	6	1	1.9	0.4	53	162	0.4
2&7	6	2	3.8 ± 1.4	1.3	110 ± 43	179 ± 39	1.0
4&5	6	3	16.8 ± 1.4	1.3	534 ± 164	327 ± 274	3.5
6	6	2	9.6 ± .4	1.4	330 ± 3	351 ± 92	2.1
D3	3	2	4.0 ± .5	0.3	100 ± 34	139 ± 8	1.1

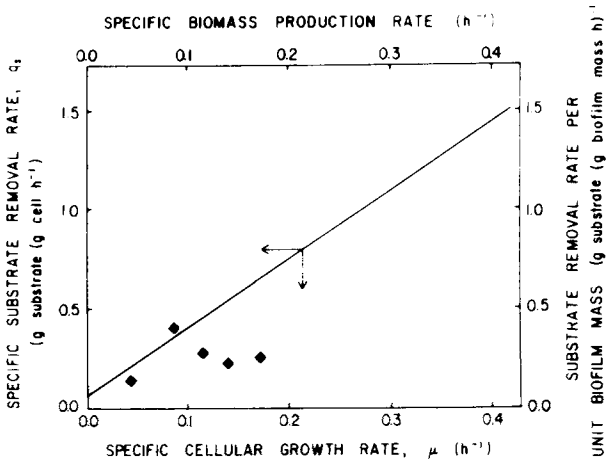

Figure 5. Comparison of substrate removal rate per biofilm mass vs. (◆) specific biomass production rate data to eq. (24) (the line is from Fig. 4).

Table VII. Characteristic coefficients for aerobic glucose metabolism by *P. aeruginosa* in a chemostat (from ref. 1).

K_s	=	2.0 g glucose carbon/m^3
μ_{max}	=	0.4 h^{-1}
$Y_{x/s}$	=	0.34 g cell carbon/g glucose carbon
$Y_{p/s}$	=	0.56 g EPS carbon/g glucose carbon
k	=	0.27 g EPS carbon/g cell carbon
k'	=	0.035 g EPS carbon/g cell carbon h

Biofilm Areal Densities

To evaluate the influence of the overall substrate flux into the biofilm on biofilm cellular density [eq. (28)], data obtained at relatively constant q_s were analyzed. All biofilms reached steady state within a relatively narrow range of specific substrate removal rates ($0.53 < q_s < 0.71$) (Tables V and VI, Fig. 4). All experiments in Tables V and VI are, therefore, included in Figure 6, which presents the fit of x_b vs. η_s data to eq. (28). The slope of eq. (28) was calculated as the inverse of the average r_s ($= 0.61^{-1}$). The error term for the residual sum of squares was not significant (at the 5% level), indicating that eq. (28) adequately predicts the dependence of x_b on η_s. A strong correlation between experimental and theoretical values imply that biofilm cellular density was proportional to substrate flux into the biofilm in all experiments. Biofilm *P. aeruginosa* density can, therefore, be estimated from reactor inflow and outflow glucose concentrations, given the same environmental conditions as in this study.

Biofilm EPS areal density was not directly proportional to substrate flux.

Biomass Detachment

The observation that biofilm mass, x_b plus p_b, did reach steady state, implies that (1) $n > 0$ in eqs. (10) and (11), or (2) the assumptions that detachment is independent of

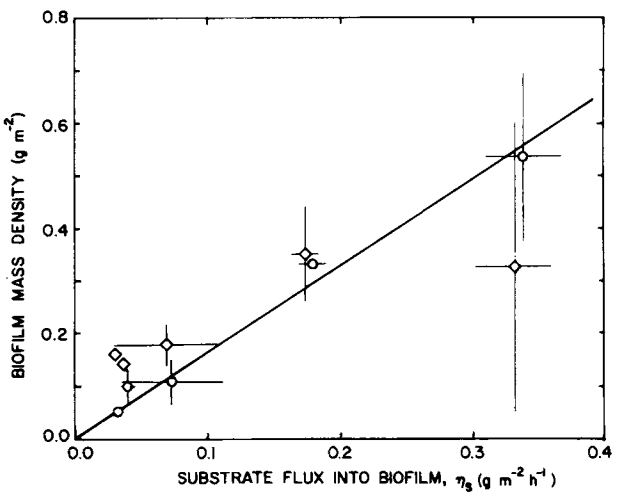

Figure 6. Fit of x_b vs. η_s (○) data to eq. (28) and (◇) p_b vs. η_s data. The slope of eq. (28) was 1.64 h. Vertical error bars represent standard error of data from two or three reactors. Horizontal error bars represent range of substrate flux.

substrate load, growth rate, or dilution rate, are not valid. Equations (29), (30), and (31) cannot be tested due to the lack of information regarding biofilm detachment kinetics.

Growth Rate

Biofilm specific cellular growth rate, μ, at steady state is presented as a function of reactor substrate concentration, s, in Figure 7. Figure 7 also includes the chemostat *P. aeruginosa* data.[1] The biofilm data, although restricted to a narrow substrate concentration range, correlate well with the chemostat data supporting the use of chemostat-derived kinetic coefficients (μ_{max} and K_s) to predict biofilm behavior. Since minimal diffusional resistance existed in these experiments,[5] reactor substrate concentration and biofilm substrate were essentially the same.

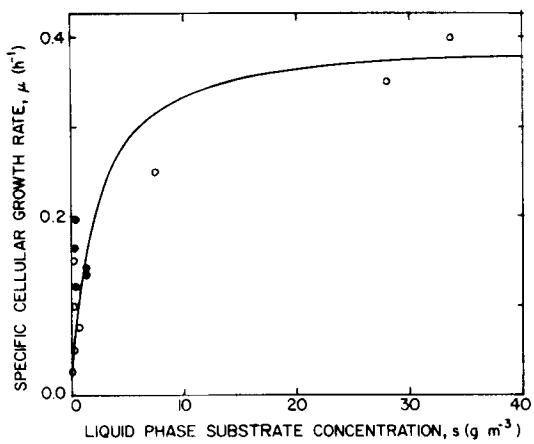

Figure 7. Comparison of steady-state specific cellular growth rate, μ, vs. reactor substrate concentration, s, data [(●) biofilm data and (○) chemostat data] to eq. (8) ($\mu_{max} = 0.4$ h^{-1} and $K_s = 2.0$ g/m^3) (from ref. 1).

CONCLUSIONS

The mathematical model, including chemostat derived stoichiometric and kinetic coefficients, compared well with steady-state biofilm data. This suggests the following:

(1) *P. aeruginosa* does not behave differently in biofilms than in suspension at steady state.

(2) Intrinsic coefficients derived in chemostats can, therefore, be used to describe steady-state *P. aeruginosa* biofilm processes.

(3) Our mathematical model adequately described aerobic glucose metabolism by steady-state *P. aeruginosa* biofilm cultures.

Further research is required to determine the validity of these conclusions for bacteria in general.

Some key factors of our process analysis resulting in these final conclusions must also be emphasized. All biofilm activity measurements were made (1) *in situ*; (2) without significant diffusional resistance (the substrate was soluble, biofilm thickness did not exceed 50 μm, biofilm cell density was relatively low, and the liquid phase was turbulent); and (3) in reactors where the submerged surface was inert (i.e., the growth surface did not affect the biofilm activity through the release or transfer of chemical compounds). The importance of the biomass separation into two components, cell and EPS mass, to the success of the mathematical model and to the increased understanding of biofilm processes must also be emphasized. Robinson and co-workers[1] clearly demonstrated the importance of increased structure in the analysis of chemostat data. It was demonstrated, for example, that maintenance requirements exhibited by *P. aeruginosa* may be attributed to EPS formation, a measured quantity. The importance of structure is even greater in the more complex biofilm systems, and may be the most important single factor resulting in a successful analysis.

The data presented in this article were excerpted mainly from the Ph.D. thesis of one of the authors (MGT). Financial support from the National Science Foundation (grant No. CPE 80-17439), the Office of Naval Research (grant No. N00014-80-C-0475), the Calgon Corporation, and the Montana State University Engineering Experiment Station is gratefully acknowledged.

NOMENCLATURE

A	wetted reactor surface area (L^2)
$\mathbf{a}$	component vector (M/L^3 or M/L^2)
$\mathbf{a}'$	transport vector ($M/L^3/t$)
$\mathbf{a}''$	transformation vector ($M/L^3/t$ or $M/L^2/t$)
D	Dilution rate (t^{-1})
EPS	extracellular polymeric substances
F	volumetric flowrate (L^3/t)
k	growth-associated polymer formation rate coefficient (M_p/M_x)
k'	non-growth associated polymer formation rate coefficient ($M_p/M_x/t$)
k_{dp}	EPS detachment coefficient (M_p^{-n}/t)
k_{dx}	cellular detachment coefficient (M_x^{-n}/t)
K_s	half-saturation coefficient (M_s/L^3)
n	reaction order (dimensionless)
p	suspended EPS carbon concentration (M_p/L^3)
p_b	biofilm EPS carbon areal density (M_p/L^2)
q_{dp}	specific EPS detachment rate (t^{-1})
q_{dx}	specific cellular detachment rate (t^{-1})
q_p	specific EPS formation rate ($M_p/M_x/t$)
q_s	specific substrate removal rate ($M_s/M_x/t$)
s	steady-state glucose carbon concentration (M_s/L^3)
s_i	influent glucose carbon concentration (M_s/L^3)
t	time (t)
V	annular reactor volume (L^3)
x	suspended cellular carbon concentration (M_x/L^3)
x_b	biofilm cellular carbon areal density (M_x/L^2)
$Y_{p/s}$	polymer yield coefficient (M_p/M_s)
$Y_{x/s}$	cellular yield coefficient (M_x/M_s)
η_s	overall substrate flux into biofilm ($M_s/L^2/t$)
μ	specific cellular growth rate (t^{-1})
μ_{max}	maximum specific cellular growth rate (t^{-1})

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